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Journal of Chromatography B, 680 (1996) 31–41

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Protein partition between the different phases comprising poly(ethylene glycol)–salt aqueous two-phase systems, hydrophobic interaction chromatography and precipitation: a generic description in terms of salting-out effects

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Abstract

The solution behaviour of selected proteins has been studied under conditions promoting precipitation, binding to mildly hydrophobic adsorbents or partition. Solvophobic theory may be used to describe these forms of protein partition. The tendency of a protein to partition therein is dependent upon surface properties of the protein solute mediated by the concentration and nature of added salts. As applied to partitioning in poly(ethylene glycol) (PEG)–salt systems this implies that linear (Brönsted) relationships apply only to proteins partitioned close to the critical point. At longer tie-line lengths protein partitioning is increasingly influenced by salting-out forces. This is confirmed by the observed behaviour of the proteins. The point at which this behaviour changes has been unambiguously defined enabling the direct comparison of phase transition of proteins during partition in all systems. The results obtained show that phase transition during adsorption and partition occur at similar concentrations of salt. This is less than that required to promote precipitation. It appears, from these limited studies, that top-phase preferring proteins are partitioned at salt concentrations above those required to cause adsorption. Proteins preferring the lower phase are partitioned at salt concentrations close to or below those required for adsorption. This raises questions regarding the solvated molecular form of the partitioned proteins and the definition of the partition coefficient.

Keywords: Partitioning; Aqueous two-phase systems; Salting-out; Proteins; Polyethylene glycol

1. Introduction

In his seminal "Nature" paper of 1958, Albertsson [1] quite correctly indicated that suitable two-phase systems for the partition of proteins were difficult to construct because proteins were easily denatured by the solvent systems which had hitherto been employed. In addition, proteins are macromolecules and have therefore a general tendency to accumulate in only one of the two phases [1]. In order to overcome the latter difficulty it is necessary to operate very close to the critical point, thus composition and operating temperature must be carefully controlled. Much earlier Brönsted [2] had demonstrated, in aqueous-organic systems, a linear relationship between the partition coefficient, k, of

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0378-4347/96/$15.00
SSDI 0378-4347(95)00448-3

colloidal inorganic particles and the distance of the system from the critical point which was dependent upon the size of the particle. Brönsted's study [3] was indeed conducted close to the critical point, suitable systems being produced by manipulation of the temperature within 0.25°C of the temperature for critical mixing [4]. Albertsson quite clearly demonstrated [1] that similar results could be obtained with proteins partitioned in polymer–polymer aqueous two-phase systems and that these relationships held further from the critical point than might be expected for the traditional solvent-based systems, presumably because of the lower interfacial tensions involved [4]. More recently Diamond and Hsu [5,6] derived a relationship from Flory–Huggins theory which was formally similar to the Brönsted relationship. However, these authors also found that a similar but empirically extended relationship better described their partitioning data particularly as applied to high molecular mass proteins for a number of PEG–dextran systems and high molecular mass PEG–salt systems. This relationship takes the form:

$$\ln k = A(w_1'' - w_1') + b(w_1'' + w_1')^2 \quad (1)$$

where w represents the weight percent concentration of the polymer, the subscript refers to PEG and the double and single primes refer to the top and bottom phase, respectively.

For some years the present authors have been attempting to produce a simple and coherent account of protein partitioning behaviour in PEG–salt aqueous two-phase systems [7–10]. A number of features of this behaviour strongly suggest that Brönsted-type relationships are incomplete descriptions and that salt-induced solution behavioural changes of proteins are a prominent feature of these systems. These features, which have previously been reported, are the following. (1) The dependence of protein partition coefficients on volume ratio at extended tie-line length in PEG–salt systems [8]. (2) The similarity of protein partitioning behaviour in these systems to their behaviour when partitioned between a mobile phase containing high concentrations of added salt and mildly hydrophobic solid-phases [9]. (3) The dependence of the protein concentration found in the lower phase of productive PEG–salt aqueous two-phase systems (containing total soluble protein from wet-milled bakers yeast) upon the salt concentration of that phase regardless of the PEG molecular mass of the system [10]. These salt-induced solution behavioural changes which bring about the partition of proteins to a mildly hydrophobic phase (which may be solid in the case of hydrophobic interaction chromatography, HIC, and salting-out or aqueous in the case of partitioning in PEG–salt systems) are further explored in the present paper. A common theoretical treatment is adopted which may usefully illuminate both the molecular origins of the observed behaviour for each type of partition and provide some basis for the rational application of these forms of partition in the design of protein purification strategies.

2. Experimental

2.1. Materials

Poly(ethylene glycol) (PEG) of relative molecular masses 1000 and 1450 was obtained from Sigma Chemicals (Poole, UK). Dipotassium hydrogen orthophosphate and potassium dihydrogen orthophosphate were of GPR grade and obtained from BDH Chemicals (Atherstone, UK). The following proteins, obtained from Sigma Chemicals, were used in some or all of the various partitioning experiments to be described: bovine serum albumin (BSA), ovalbumin, lysozyme, cytochrome *c*, ribonuclease A, myoglobin, α-amylase, α-chymotrypsin and bovine methaemoglobin. Some physical properties of these solutes may be found tabulated in Ref. [9].

2.2. Preparation of the phase systems and partitioning of proteins

Binodal curves were determined by the cloud point method [11] from stock solutions of 50% (w/w) PEG and 30% (w/w) potassium phosphate. Phosphate solutions were formulated at a constant weight ratio of 18:7 (K_2HPO_4/KH_2PO_4) [9]. Tie lines were estimated from the known relationship [12] between the volume ratio and the intersection by the tie line of the overall system composition and the

binodal curve. All experimental systems for partitioning were selected to have a volume ratio of unity. From well mixed stock solutions of the selected systems, aliquots of known weight, having a volume of 10 or 5 ml, were withdrawn. Accurately weighed, freeze-dried protein samples were then added to a final overall concentration of 3 mg/ml. Dissolution of the protein took place over 12 h at 25°C and phases were separated at 2000 *g* for 5 min. Phases were carefully sampled and, after suitable dilution, a single determination of spectrophotometric absorbance was used to determine the equilibrium concentrations.

2.3. Hydrophobic interaction chromatography of proteins

Hydrophobic interaction chromatography performed as described previously [9] used a Bio-Rad PEG 300-10 column (Bio-Rad RSL, Belgium) 150 mm long and 4.6 mm in diameter, packed with 10 μm silica particles of 30 nm pore size and derivatised with low molecular-mass PEG (molecular mass 1000). In some experiments a polymeric methyl column, 50×7.8 mm in diameter (Bio-Gel MP7, Bio-Rad Laboratories, Hemel Hempstead, UK), having 7 μm particles of 80 nm pore size was used under identical conditions. Isocratic retention times of 20-μl injections of each protein at a concentration of 1.5 to 3 mg/ml were measured for increasing salt concentrations in the running buffer (between 2 and 30% w/w phosphate) at a flow-rate of 0.2 ml/min and a temperature of 25°C. The phosphate concentrations in the running buffer had the same weight ratio as for the partitioning experiments.

2.4. Precipitation of proteins

Solubility of selected proteins in increasing concentrations of the same phosphate buffer solution as used above was determined. Aliquots of a protein stock solution, a salt stock solution and water were mixed in 1.5-ml tubes to yield salt concentrations in the range 0.5–4.5 molal and final protein concentrations (unprecipitated) of 7 mg/ml except in the case of cytochrome *c* when the final protein concentration was 3 mg/ml. The tubes were well mixed using a rotamixer and centrifuged at 10 000 *g* for 10 min after standing for 15 min. Samples were pipetted carefully from the liquid-phase, especially under circumstances in which floating precipitates were formed, and protein concentration was estimated by absorbance at 280 nm against reagent blanks.

3. Results and discussion

Kim derived a model of partitioning in PEG–salt aqueous two-phase systems [13] based upon cavity theory to describe the effects of salt on protein solution behaviour and upon excluded volume considerations [14] to describe the effects of PEG. Briefly, the partition coefficient is equated to the relative solubility of the protein in the two coexisting phases. For the bottom, salt-rich, phase protein solubility is described by a simple salting-out relationship [15].

$$\ln C = \ln C_0 + \beta + \Lambda m - \Omega\sigma m \quad (2)$$

in which the protein solubility (C) is related to its intrinsic solubility (C_0) by a salting in constant (β) derived from Debye–Hückel theory, a salt concentration (m) dependent term (Λ) derived from Kirkwoods treatment of proteins as dipolar ions applicable at high salt concentration and a salting-out term (Ω) which is related to the hydrophobic surface area of the solute and depends on the specific surface tension increment (σ) of the salt.

For the top, PEG-rich, phase protein solubility is accounted for by considering that the salt in the PEG-rich phase is excluded from the polymer surface and its concentration thereby increased in the PEG-free solvent of that phase. A further term accounting for any additional exclusion of proteins by PEG is also included.

$$\ln C = \ln C_0 + \beta + \Lambda - \Omega\sigma m_{sf} - \alpha_b m_p \quad (3)$$

in which the newly introduced terms represent: for α_b the excluded volume of PEG for protein, m_p the concentration of PEG and m_{sf} is the effective concentration of salt in the volume of the phase

which is not occupied by PEG. The latter is related to the actual concentration in the phase by:

$$m_{sf} = m_s \exp(\alpha_s m_p) \quad (4)$$

where m_s is the apparent salt concentration in the phase, α_s is the excluded volume of PEG for salt. The latter is determined directly from the phase diagram in the form of the log solubility of salt in PEG [13]. The excluded volume of PEG for proteins is determined from the decrease in their solubility with salt concentration determined at different levels of PEG addition [13].

Fig. 1a shows the phase diagram of a potassium phosphate PEG 1450 system and, derived from this, Fig. 1b shows the change in composition of the lower phase of this system as the tie-line length increases. It is immediately apparent that the form of Kim's model given above cannot successfully predict partition approaching the critical point as the compositions of the two phases converge. At the critical point the compositions are identical and yet the model treats them differently. In this form the partition coefficient cannot, therefore, approach unity as the critical point is approached. The simplest way of correcting this deficiency is to replace the description used for the bottom phase with that for the top phase. At extended tie-line length the two descriptions will effectively revert to their original forms as may be judged from Fig. 1. Fig. 1b also shows that close to the critical point the bottom phase becomes rapidly depleted in PEG but shows only slow rise in phosphate concentration whilst later the increase in phosphate concentration is more rapid. The results to be presented here suggest that the interplay of molecular forces resulting from these compositional changes give rise to protein partitioning behaviour which may at first conform to the Brönsted relation (being driven largely by exclusion forces) but is later determined by strong salting-out forces. The system composition at which this occurs varies for different proteins.

Fig. 2a shows the results obtained by applying this model with salting-out parameters varied about values typical of the partition of total yeast protein in PEG–phosphate aqueous two-phase systems [10]. A graphical representation of the data from which these values were derived may be found in Ref. [10]. The

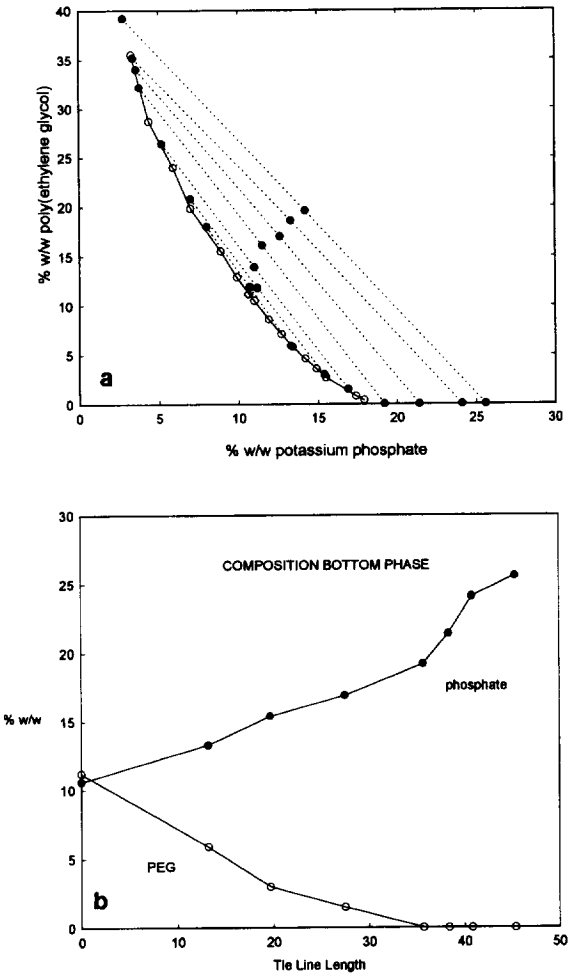

Fig. 1. (a) Phase diagram of a PEG 1450 potassium phosphate phase system showing experimentally determined binodal (○) and tie lines (●). (b) Composition of the bottom phase of the PEG 1450 potassium phosphate system as a function of tie-line length. Symbols: (●), phosphate concentration and (○), PEG concentration.

excluded volume of PEG for salt parameters were estimated from the phase diagram [13]. The maximum intrinsic solubility ($\ln C_0 + \beta$) was arbitrarily assigned an identical value of 8 in each case. Here the excluded volume of PEG remains an adjustable parameter set below known values for higher molecular masses of PEG [11] but greater than zero. Since proteins are often quite soluble in low molecular mass PEG accurate values are rather difficult to

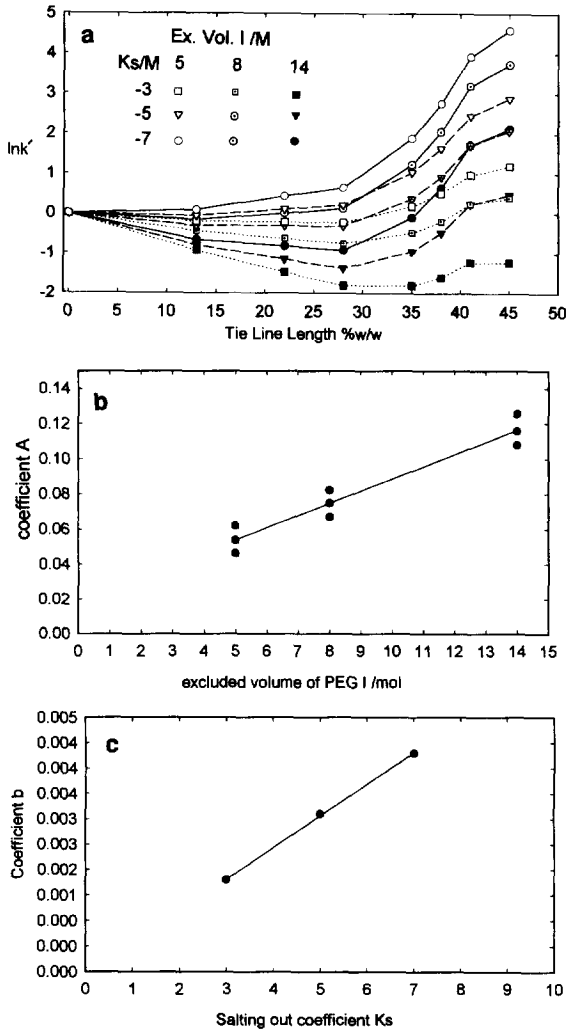

Fig. 2. (a) Simulation of the partitioning behaviour of proteins in PEG–salt aqueous two-phase systems using the modified model of Kim. Partition coefficient as a function of tie-line length. The correspondence of the symbols used to the variable parameters of the model are indicated in the table. (b) The dependence of the coefficient *A* (Diamond and Hsu, Eq. 1) on the excluded volume of PEG used in the generation of (a). (c) The dependence of the coefficient *b* (Diamond and Hsu, Eq. 1) on the salting-out constant used in the generation of (a).

obtain. The actual parameters used are shown in the figure. With these parameters a family of curves is produced (Fig. 2a) which vary from conditions which result in partition to the upper phase at longer tie-line length and conditions which favour partition to the lower phase throughout. Conditions may also be seen in which the partition coefficient is at first lower than for other starting conditions but eventually exceeds them as the tie-line length increases. With the parameters used a range of partitioning behaviours has been reproduced but it should be noted that no strongly top-phase preferring proteins are visible. The results from this model have been treated according to the empirical relationship of Diamond and Hsu [6] (Eq. 1) to yield the coefficients. Fig. 2b and Fig. 2c show that the coefficient *A* is strongly related to the excluded volume of PEG parameter but also contains information relating to the salting-out strength as evidenced by the residual scatter about the regression whereas the coefficient *b* appears to be related to the salting-out strength alone. In the present context the latter seems particularly significant and is approximately confirmed by the limited data available on the partition and precipitation of model proteins (data not shown) [16].

Experimental partitioning data for selected model proteins is shown in Fig. 3a linearised in accordance with the empirical relationship of Diamond and Hsu (Eq. 1). (The complete experimental data, obtained as outlined in Section 2, may be found in Ref. [9].) From this data the coefficients *A* and *b* may be obtained from the intercept and slope of the data shown in Fig. 3a. Whilst this data was obtained at some remove from the critical point, in order to study salting-out effects, the coefficients may be used to interpolate the data between the critical point and the shortest tie-line length used experimentally [9] as shown in Fig. 3b. This represents a qualitatively good description of the observed and inferred (close to the critical point) partitioning behaviour [9]. At the critical point all proteins have a partition coefficient of unity and for the majority the partition coefficient then falls as the tie-line length increases. Interestingly we have previously shown [8] that partitioning data obtained under near critical conditions may depart from expectations due, presumably, to the relatively delicate balance of physico-chemical forces existing in this region which may be disturbed by the addition of rather small amounts of solute. Ultimately the partition coefficient increases from a minimum which depends upon the relative magnitude of the two coefficients. By

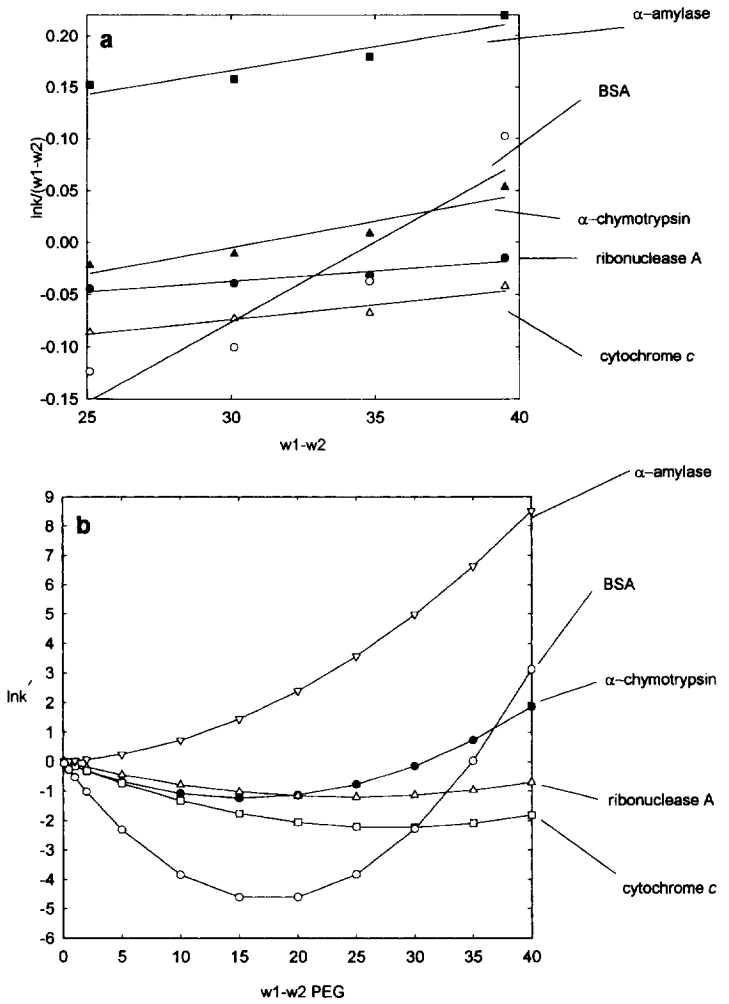

Fig. 3. (a) Partition of selected model proteins in a PEG 1450 potassium phosphate aqueous two-phase system plotted according to a rearrangement of Eq. 1; $w_1 - w_2$ represents the PEG concentration difference (% w/w) between the co-existing phases. (b) The distribution of selected model proteins as a function of the PEG concentration difference (% w/w) between the phases in a PEG 1450 potassium phosphate system. This distribution is calculated from the coefficients determined from Eq. 1 in (a).

setting the first derivative of Eq. 1 to zero (i.e. zero slope) the point at which this occurs may be unambiguously defined and we denote it as the critical salt concentration for the phase transition from the lower to the upper phase.

Fig. 4 illustrates the salting-out behaviour from potassium phosphate solutions of a selection of the model proteins whose partitioning behaviour has been described above [9]. The salting-out behaviour of these proteins may be described by the same theoretical relationships [15] as were used to describe salting-out from the lower phase (Eq. 2 above). Examination of Fig. 3b and Fig. 4 reveals that a clear relationship exists between the order in which these proteins salt-out in relation to salt concentration and the magnitude of their partition coefficients at extended tie-line length when partitioned in low molecular mass PEG–phosphate aqueous two-phase systems (see Fig. 3). However, the slopes of the salting-out data (K_s) appear not to

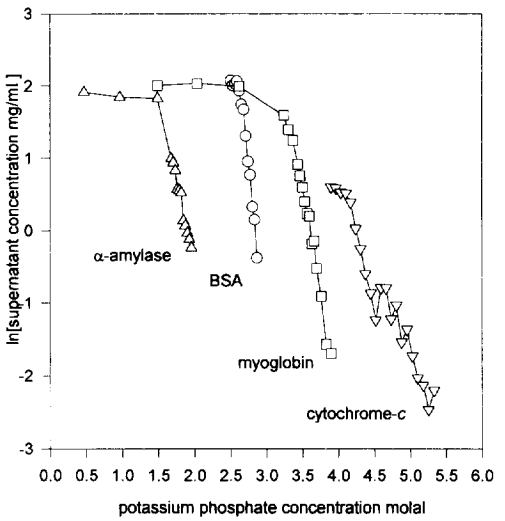

Fig. 4. Precipitation of selected proteins with potassium phosphate as described in Section 2. ln (supernatant concentration) as a function of the molality of the potassium phosphate used.

be at all closely related to the partition coefficient (compare the slopes of BSA and α-amylase). The salting-out constant K_s is partially dependent on the intrinsic solubility ($\ln C_0 + \beta$) which is widely different for different proteins and can result in large differences in k_s. The use of the critical salt concentration necessary to bring about precipitation (m^*) has been suggested [17,18] as a means of characterising the salt-induced solution properties of proteins. This parameter is estimated from a plot of the precipitation data (Fig. 4) according to Eq. 2. The data may then be fitted by two straight line segments, one from the starting concentration to the onset of precipitation and the other during the course of precipitation which is equivalent to k_s. The intersection of these two lines gives m^* which we denote the critical concentration for the phase transition from the liquid- to the solid-phase. This parameter, obtained in this way, is self evidently concentration dependent; however, it is claimed that this is not the case when the parameter is estimated at infinite solute dilution [17]. Since in the present case the protein concentrations used are far from their maximum solubility and broadly similar for all three partitioning systems it seems, at least temporarily, acceptable to use this parameter as a measure of the susceptibility of each protein to salting-out forces.

Fig. 5 shows the close relationship which exists between the critical concentration of each phase transition. It is necessary to admit that the data for the partition coefficient of α-amylase in the PEG 1000 system shows an asymptotic maximum rather than a minimum (Examination of Fig. 3b may help to clarify this point.) Since this protein strongly prefers the upper phase it has arbitrarily been assigned a critical point for the phase transition of zero % w/w tie-line length in Fig. 5 and also in Fig. 8a and Fig. 9. This is very close to the value actually measured in the PEG 1450 system (−2.75% w/w TLL) but differs considerably from the real value obtained (36.5% w/w TLL). Despite this it appears that bottom-phase preferring proteins such as cytochrome *c* are generally partitioned a long way from the critical concentration of the liquid–solid-phase transition during salt precipitation. Proteins preferring the top phase are partitioned very much closer to this critical concentration in these low molecular mass PEG–salt systems.

The binding of proteins to mildly hydrophobic

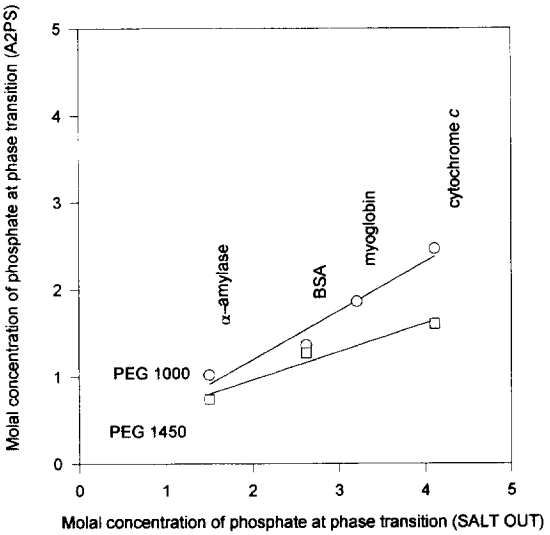

Fig. 5. The critical molal phosphate concentration of the phase transition A2PS (see Section 3) compared to the critical concentration of the liquid–solid phase transition during salting-out.

solid-phases has also been subject to similar theoretical treatment [15] to that given above for precipitation and partition. In this case:

$$\ln D = \ln D_0 - \beta' - \Lambda' m + \Omega' \sigma m \quad (5)$$

where D is the chromatographic retention parameter defined as:

$$D = \frac{V_e - V_0}{V_0}$$

in which V_e is the measured isocratic elution volume and V_0 is the elution volume in low salt (2% w/w phosphate). Other terms are as defined above (Eq. 2) except that the primes denote that what is of importance is the free-energy change associated with the binding of a fraction of the protein surface to the ligand. It is tacitly assumed that the same forces are involved in HIC as in precipitation but that in HIC these represent some fraction of those required to bring about complete precipitation. Notice also that the nature of the experimental procedure has the result that certain terms change sign as compared to their use in the description of precipitation.

Fig. 6 shows the isocratic elution times of several proteins on the Bio-Gel MP7 $-O-CH_3$ column. It is apparent that the resolution of this column is low particularly for proteins which elute in the mid range of salt concentration. This may be due to an unfavourable aspect ratio of this column when run in isocratic mode. Comparison of Fig. 3b and Fig. 4 to Fig. 6 shows that the order of elution from the column in response to salt concentration is similar to both partition and salting-out. We have previously shown that there is little detailed correspondence between the chromatographic retention parameter and the partition coefficient obtained in aqueous two-phase systems [9]. However, this data may be expressed as the chromatographic retention parameter ($\ln D$) versus salt concentration of the mobile phase (data not shown) in conformity with Eq. 5 and once again the intersection of two line segments covering the regions where the protein is only weakly retained and the region where the protein is strongly retained may be obtained. This yields a measure analogous to m^* for salt precipitation which we denote the critical concentration for phase transition from liquid- to solid-phase (HIC). Using this measure the results may be directly compared to results previously obtained on a PEG–silica column [9]. These are shown in Fig. 7. The comparatively poor resolution of the $-O-CH_3$ column is highlighted but note that the aspect ratio of these two columns is rather different (see methods). Taken at face value Fig. 7 implies that the $-O-CH_3$ column is more hydrophobic than the PEG 1000 column since a slightly greater amount of salt is required to initiate the phase transition on the PEG column. This is even more apparent when chromatographic retention parameters are compared (data not shown). However, since nothing is known of the ligand density on these commercial column packings such statements are not valid. Perhaps the best that can be said is that the columns are of similar hydrophobicity which, when set in the context of the effect of increasing the alkyl chain length on the performance of hydrophobic interaction chromatography, suggests that both these solid-phases may indeed be described as mildly hydrophobic.

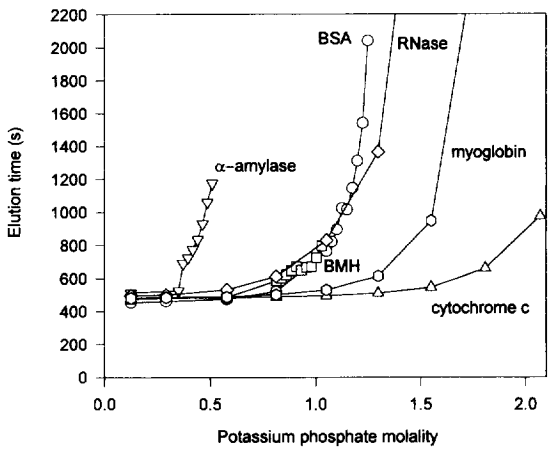

Fig. 6. Isocratic elution profiles of selected proteins as a function of mobile-phase phosphate molality on a Bio-Gel MP7 methyl HIC column 50×7.8 mm I.D. Abbreviations: BMH=bovine methaemoglobin; BSA=bovine serum albumin; RNase=ribonuclease A.

Fig. 8 shows the molality of the critical phase transition for HIC determined on the PEG–silica column [9] compared to the molality of the critical phase transition as determined for two PEG–phos-

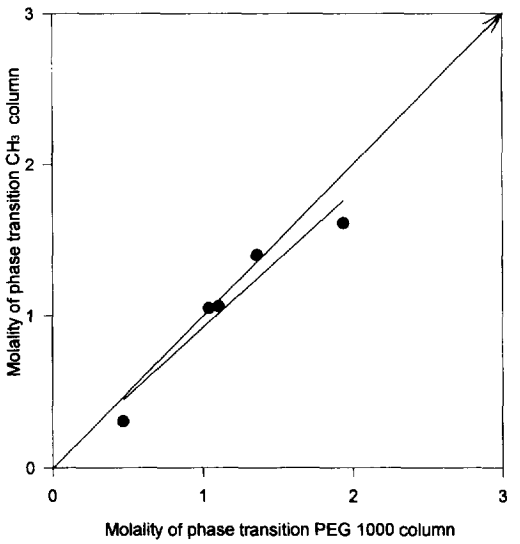

Fig. 7. The critical molality of the phase transition from liquid- to solid-phase (see Section 3) of the Bio-Gel MP7 methyl column (50×7.8 mm I.D.) compared to the Bio-Rad PEG 300-10 column (150×4.6 mm I.D.).

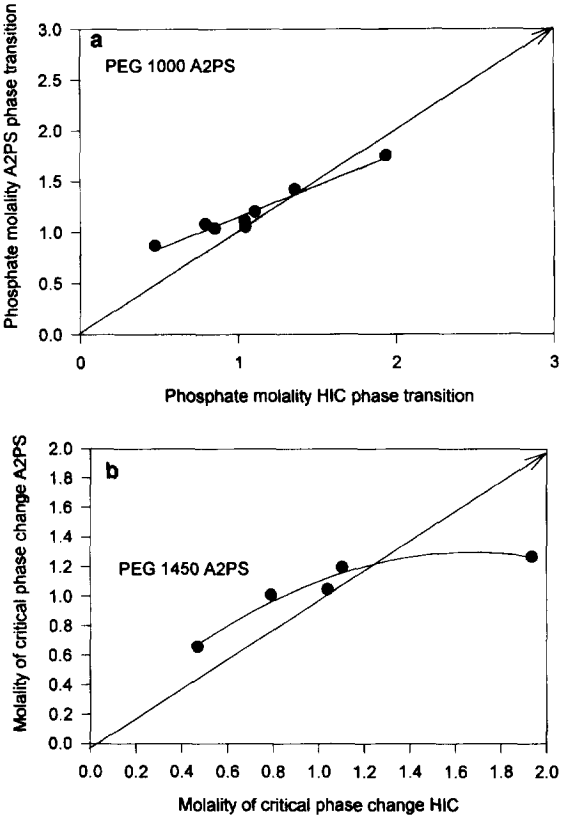

Fig. 8. The critical molality of the phase transition in A2PS compared to the critical molality of the phase transition during HIC on the Bio-Rad PEG 300-10 column (PEG 1000 ligand): (a) for PEG 1000 systems; (b) for PEG 1450 systems.

phate partitioning systems comprising PEG of molecular mass 1000 and 1450. Bearing in mind the aforementioned proviso regarding the α-amylase data in the PEG 1000 system it appears that proteins that prefer the upper phase or which can be induced, by increase in salt concentration, to partition to the upper phase are partitioned at salt concentrations above those required to bring about binding to a solid-phase. Proteins preferring the lower phase are partitioned at concentrations close to or below those required to bring about adsorption to a mildly hydrophobic solid-phase. It seems that chromatography on this type of column can yield valuable information of use in the design of partitioning steps for a relatively modest investment in experimental work.

Finally, Fig. 9 compares the critical molality of the phase transition for each process for several proteins spanning the widest available range of behaviours. It is apparent that the critical phase transition occurs at rather similar concentrations of phosphate for partition and HIC. In the case of salting-out the concentration of salt required to bring about the phase transition is rather higher. This is particularly so for proteins which are difficult to salt-out and generally partition to the lower phase. Other proteins appear to salt-out rather more easily and are generally found or may be driven by increase in tie-line length to the upper phase. It is interesting to note that for two proteins the critical phase transition for the PEG 1000 system occurs at higher molality than the PEG 1450 system. This suggests that the PEG 1450 system is the more hydrophobic, which is reasonable. However, the partition coefficient of proteins is expected to decrease between these two molecular masses [7] which serves to emphasise the neglect of PEG exclusion forces in this treatment. It is hoped to examine these features in a subsequent publication.

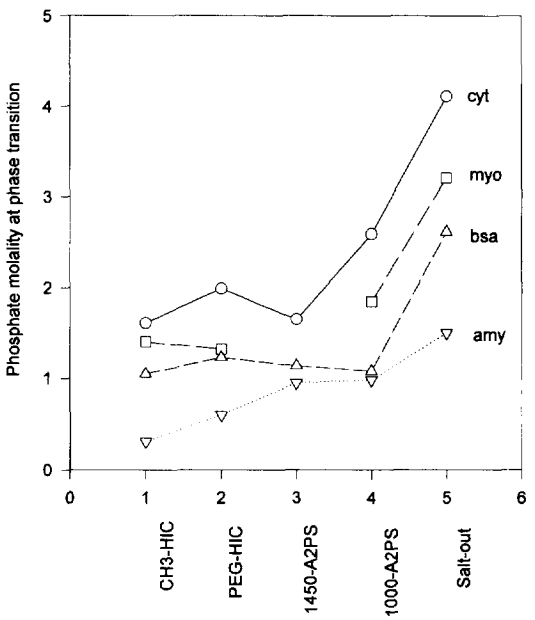

Fig. 9. The critical molality for the phase transition (liquid–liquid or liquid–solid) for each of the partitioning systems examined. CH_3-HIC indicates the Bio-Rad MP7 column; 1450-A2PS indicates partitioning in the PEG 1450 potassium phosphate system; 1000-A2PS indicates partitioning in the PEG 1000 potassium phosphate system; PEG-HIC indicates results obtained on the Bio-Rad PEG 300-10 column; salt-out indicates the phase transition during salting-out experiments. Abbreviations: amy=α-amylase; bsa=bovine serum albumin; myo=myoglobin; cyt=cytochrome *c*.

4. Conclusions

It has been shown that partition of proteins in PEG–salt aqueous two-phase systems is strongly influenced by their solubility in concentrated solutions of salts. Theoretical descriptions of protein partitioning must take this into account particularly in attempting to deal with the effect of protein charged state on partitioning behaviour. It appears that PEG–salt systems may be placed in a continuum of salt-induced partitioning behaviour approximately bracketed by HIC and salting-out. It is to be hoped that this seemingly trivial observation will encourage the adoption of PEG–salt partition in protein recovery schemes. Knowledge of their interrelatedness also highlights the advantages and pitfalls to be expected during integration of these processes and indicates, albeit in a broad way, the consequences of such integration in a process design context. The theoretical treatment which has been used to illustrate this similarity contains within it the implication of a dehydration of the protein surface as a result of the increase in surface tension of the salt solution. This may be thought obvious in the development of a protein precipitate and may also be thought to occur on the binding of a protein to a ligand during HIC. That the salt concentrations are so similar between HIC and partition may be thought to imply that partition to the PEG phase of an aqueous two-phase system also involves a hydrational change at the protein surface. In turn this seems to imply that there may be different species present in either phase in which case the simplest form of the partition coefficient cannot be applied. Other observations lend support to this hypothesis, namely that beyond the phase transition partition coefficients become dependent on volume ratio [8]. In addition, the protein concentration in the lower phase of productive aqueous two-phase systems is dependent only on the salt concentration of the phase and not at all on the molecular mass of PEG employed [10].

For the future a more complete understanding of protein partition in PEG–salt systems awaits the dissection of the relatively complex interaction of PEG with salts and proteins which is implied in Kim's model for the behaviour of the upper phase. For example, the excluded volume of PEG varies from salt to salt [19] and from protein to protein (see Fig. 3b). In addition, the presence of PEG may affect the dielectric constant of the medium [16]. The lack of publicity which has been given to this model of partitioning is curious. Certainly the complexity introduced by the separate salt and protein exclusion terms is interesting and reflects at least in a qualitative way the actual complexity of PEG precipitation [16]. It seems likely that experimental accounts in the literature on PEG precipitation of proteins are relatively incomplete.

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